Journal of Chromatography, 614 (1993) 109–116
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6742

Direct determination of ibuprofen and ibuprofen acyl glucuronide in plasma by high-performance liquid chromatography using solid-phase extraction☆

Marta Castillo

College of Pharmacy, University of Texas at Austin, Austin, TX 78712 (USA)

Philip C. Smith*

School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599 (USA)

(First received October 20th, 1992; revised manuscript received January 4th, 1993)

ABSTRACT

A method for the simultaneous determination of ibuprofen and its labile, reactive metabolite, ibuprofen acyl glucuronide, in plasma is described. Reversed-phase high-performance liquid chromatography (HPLC) employed a C_{18} column using methanol–10 m*M* trifluoroacetic acid as the mobile phase with ultraviolet detection at 225 or 214 nm. It is essential that blood is handled rapidly and plasma is acidified upon collection prior to freezing. Plasma samples first are deproteinated with acetonitrile, the supernatant is diluted with phosphate buffer, and ibuprofen, ibuprofen glucuronide, and ibufenac (internal standard) are initially isolated by solid-phase extraction on C_{18} cartridges. Upon elution, the residue is evaporated, dissolved and injected onto the HPLC system. Recovery is 94 ± 8 and 70 ± 9% for ibuprofen glucuronide and ibuprofen, respectively. The measurable concentration range is linear from 0.1 to 10 μg/ml for ibuprofen glucuronide and from 0.5 to 100 μg/ml for ibuprofen. The method is satisfactory for the analysis of ibuprofen and ibuprofen glucuronide from pharmacokinetic studies in humans. The direct determination of ibuprofen glucuronide allows accurate measurement of this conjugate at low levels relative to the parent compound, ibuprofen, a distinct advantage compared to previously employed indirect methods.

INTRODUCTION

Conjugation of carboxylic acids with glucuronic acid to yield an acyl or ester glucuronide is a major metabolic route for the biotransformation of many drugs, endogenous compounds and xenobiotics [1]. Some acyl glucuronides bind covalently to plasma proteins as shown by *in vitro* and *in vivo* studies for glucuronides of bilirubin [2,3], zomepirac [4], tolmetin [5], diflunisal [6] and probenecid [7]. In recent years, an increasing interest in drugs excreted as acyl glucuronides has emerged, because they are potentially reactive metabolites under physiological conditions; therefore, there is the necessity to study the disposition of acyl glucuronides in animal and man in order to understand various aspects of their biological action. However, since acyl glucuronides are most frequently highly polar, water-soluble and frequently unstable under physiological conditions, precautions have to be considered during their analysis and characterization [8,9].

Ibuprofen (IBP) is a widely used over-the-

* Corresponding author.

☆ This work was presented in part at the American Association of Pharmaceutical Scientists 6th Annual Meeting, Washington, D.C., November 1991.

0378-4347/93/$06.00

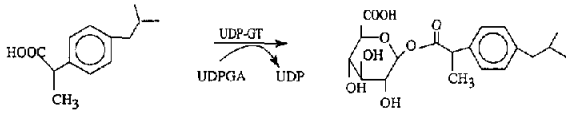

Fig. 1. Metabolism of IBP to IBPG via uridine diphosphate glucuronyl transferase.

counter non-steroidal anti-inflammatory drug (NSAID). After the administration of IBP to humans, 13% of the dose is excreted in urine as its acyl glucuronide (IBPG) (Fig. 1) [10]. Several methods have been published for determination of IBP in plasma [11–19] and IBP and its conjugates in urine which include the indirect analysis of IBPG, obtained by measuring IBP prior to and after hydrolysis of the labile acyl glucuronide with base or enzyme treatment [13,19,20]. Because the indirect methods require the measurement of a difference between two assays, the results obtained are less accurate than those obtained from direct methods, especially at low levels of the glucuronide relative to the parent compound. A separation of IBP and IBPG using ion-pairing with cethexonium and UV detection at 278 nm was reported for the analysis of high concentrations of IBPG in urine [21], however, no validation was provided. Our interest in studying the disposition and reactivity of IBPG in plasma required an analytical method for the direct measurement of IBPG and IBP in plasma at concentrations below 1 μg/ml.

In the present work, a direct method for the simultaneous determination of IBPG and IBP in plasma was developed by reversed-phase HPLC following a solid-phase extraction. Furthermore, a method for the isolation and purification of IBPG from human urine is described.

EXPERIMENTAL

Reagents

IBP was supplied by MCB Reagents (Cincinnati, OH, USA) and ibufenac was obtained by a generous contribution from Boots (UK). IBPG was obtained by extraction and purification from urine using preparative HPLC, as described below. β-Glucuronidase, type B-10 from bovine liver, was obtained from Sigma (St. Louis, MO, USA), and D-saccharic acid 1,4-lactone was from Sigma. HPLC-grade acetonitrile and methanol were purchased from EM Science (Gibbstown, NJ, USA) and Mallinckrodt (Paris, KY, USA), respectively. All other chemicals employed were reagent grade.

Equipment

The preparative HPLC system used consisted of a Pharmacia LKB 2150 pump (Bromma, Sweden), a manual injector connected to a 1-ml loop, an LKB 2141 variable-wavelength UV detector set at 225 nm, an Econosil ODS reversed-phase column (250 mm × 10 mm I.D. with 10-μm particles) (Deerfield, IL, USA) and methanol–10 m*M* acetic acid (pH 3) (65:35, v/v) at 3.5 ml/min as the mobile phase. Chromatograms were recorded on a Hewlett-Packard 3396 Series II integrator (Palo Alto, CA, USA). The analytical HPLC system used consisted of a Bio-Rad Model AS-100 HRLC automatic injector, a Hewlett-Packard Series 1050 UV detector set at 225 nm, an Axxiom ODS reversed-phase column (15 cm × 4.6 mm I.D. with 5-μm particles) (Springfield, VA, USA), a Brownlee Labs. RP-18 guard column (15 mm × 3.2 mm I.D., with 7-μm particles) (San Jose, CA, USA) and methanol–10 m*M* trifluoroacetic acid (TFA), pH 2.2 (57:43, v/v) at 1.2 ml/min as the mobile phase. The assay was also later evaluated using a Waters 441 fixed-wavelength UV detector operated at 214 nm (Milford, MA, USA).

Isolation and purification of ibuprofen glucuronide

Urine from a normal healthy subject was collected for 4 h after oral administration of a 800-mg dose of IBP, then immediately adjusted to pH 3 with acetic acid. After filtration of the urine, aliquots of 60 ml were passed through a Sep Pak C_{18} cartridge (size 360 mg) by using an infusion pump at a rate of 1 ml/min. The cartridge was washed with 3 ml of 10 m*M* TFA, pH 2.2, followed by 3 ml of acetonitrile–10 m*M* TFA, pH 2.2 (80:20, v/v) to elute and collect the IBPG-rich fraction. The solvent of this fraction was evap-

orated with a rotary evaporator at 37°C. Urine extract was redissolved in 20 ml of methanol–water (40:60, v/v), adjusted to pH 3 with acetic acid and then the glucuronide was purified further by the use of preparative HPLC. Aliquots of 600 μl of reconstituted urine extract were injected onto the preparative HPLC column and fractions were collected every 30 s for 30 min. The collected fractions containing IBPG were pooled, evaporated at 37°C to remove methanol, then lyophilized.

The identity of IBPG was confirmed by release of IBP after hydrolysis with strong base. Aliquots of the IBPG fractions from preparative chromatography were adjusted to pH 12 with 5 *M* sodium hydroxide, then heated at 80°C for 1 h. The samples were neutralized with acetic acid until pH 3, then injected onto the analytical HPLC system. Standardization of IBPG was done by comparing the UV response on HPLC of three different dilutions of a IBPG stock solution before and after the hydrolysis with 5 *M* NaOH at 80°C for 1 h. The areas obtained from these samples were compared with standard solutions of IBP. To confirm that the conjugate was a β-1-glucuronide, a solution of 20 μg/ml glucuronide in 150 m*M* phosphate buffer at pH 5 was incubated with β-glucuronidase (5000 U/ml) at 37°C for 30 min; as a control β-glucuronidase incubation was also done with 20 m*M* D-saccharic acid 1,4-lactone to inhibit the enzyme. After the incubation, an aliquot of 200 μl was taken, then 100 μl of acetonitrile were added to stop the enzymic cleavage. The sample was then analyzed by HPLC. The identity of the metabolite was confirmed by FAB-MS.

Sample preparation

A stock solution of 1 mg/ml IBP was prepared in methanol. Stock solutions of IBPG were prepared by dissolving 19.5 mg of IBP equivalents into 10 ml of acetonitrile–1% acetic acid (25:75, v/v), pH 2. A 1 mg/ml stock solution of ibufenac as the internal standard (I.S.) was prepared in methanol and by appropriate dilution with methanol, a standard solution of 80 μg/ml was obtained. When stored at −20°C, no apparent degradation of these stock solutions was noted over three months. Standard curves at concentrations of 0.5, 1, 2.5, 5, 10, 25, 50 and 100 μg/ml for IBP and 0.2, 0.5, 1, 2, 5 and 10 μg/ml for IBPG were prepared (concentrations are stated in IBP equivalents throughout the text). The standard curves were prepared with blank human plasma buffered to pH 2 with phosphoric acid (20 μl of 43% phosphoric acid per ml plasma) to prevent pH-dependent degradation of IBPG.

Each aliquot of 0.5 ml plasma was spiked with 25 μl of I.S. solution (final concentration 4 μg/ml), mixed by vortex-mixing for 30 s, then equilibrated for 10 min at room temperature. Acetonitrile (1 ml) was then added and protein precipitation was done by vortex-mixing for 30 s. Following centrifugation in a clinical centrifuge at 1200 *g*, the supernatant was removed into a screw-top tube and mixed with 8.5 ml of 10 m*M* phosphoric acid, pH 2. Extraction of drug and metabolite from plasma was done by solid-phase extraction. The extraction cartridges (Bond-Elut, C_{18}, size 100 mg) were prewetted sequentially with 4 ml each of methanol, water, then 10 m*M* phosphoric acid, before loading the diluted supernatant from the plasma samples. The sample was loaded with vacuum gravity flow onto a Bond-Elut cartridge, then the column was washed with 2 ml of acetonitrile–10 m*M* phosphoric acid (20:80, v/v). The cartridges were centrifuged in culture tubes at 1800 *g* for 3 min to remove liquid completely, transferred to clean culture tubes, and IBP and IBPG were eluted by gravity with 1 ml of acetonitrile–10 m*M* phosphoric acid (50:50, v/v), with a final centrifugation for 3 min at 1800 *g* to collect all the eluent. The eluent was evaporated to dryness under a stream of nitrogen gas at 37°C, the residue was reconstituted into 400 μl of acetonitrile–methanol–1% acetic acid (pH 3) (10:15:75, v/v), and the sample was dissolved by ultrasonic bath for 3 min and vortex-mixing for 1 min. Finally, 100 μl were injected onto the HPLC system with the chromatography conditions as described above for the analytical HPLC system.

Selectivity and extraction efficiency

In order to determine if there were interfering peaks from endogenous compounds present in plasma samples, blank plasma and plasma spiked with IBPG, IBP and I.S. were prepared using the extraction technique described above, then analyzed by HPLC. The assay specificity was tested by analyzing blank plasma spiked with aspirin, acetaminophen or salicylic acid at 100 μg/ml. The chromatograms of these spiked plasma samples were compared to those of respective standard solutions of these over-the-counter drugs.

Recovery values were evaluated by comparing the drug/ibufenac ratio from spiked plasma samples (IBPG 0.1–10 μg/ml and IBP 0.5–50 μg/ml) obtained by solid-phase extraction to unextracted standard curves prepared in aqueous solution. Ibufenac was used here as an external standard to determine the recoveries.

Application

The application of the method was demonstrated after oral administration of 800 mg of IBP to one human volunteer. Blood samples of 10 ml were collected until 7 h after the drug administration. In order to minimize the degradation of IBPG, blood samples were immediately cooled on ice after collection, plasma was obtained in a refrigerated centrifuge at 1800 *g* for 5 min, the pH was adjusted to 2–4 with 43% phosphoric acid (20 μl/ml of plasma) and then frozen at −20°C prior to analysis. Previous *in vitro* studies indicated that this procedure minimized degradation of labile IBPG to less than 2%. Quantitation of the samples was done from a weighted (1/concentration) least-squares regression analysis of the standard curve.

RESULTS

Isolation of ibuprofen glucuronide

The IBPG recovered and purified from human urine represented about 4% of the dose administered. IBPG was 95% β-1 conjugate with the remainder present as isomeric conjugates formed by intramolecular acyl migration that are resistant to β-glucuronidase cleavage, but susceptible to cleavage by strong base [22]. FAB-MS confirmed the identity of the metabolite. Positive ion FAB-MS performed in thioglycerol provided confirmatory identification of IBPG with $M + H^+$ (m/z 383) and loss of glucuronic acid to yield $IBP + H^+$ (m/z 207).

Selectivity

Fig. 2 includes chromatograms of blank plas-

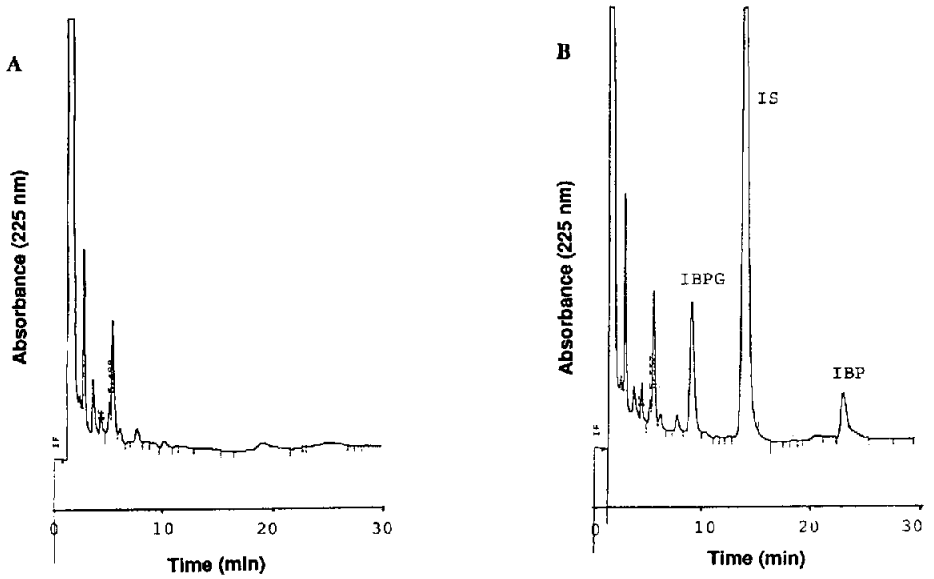

Fig. 2. Chromatograms of (A) blank plasma and (B) plasma spiked with 0.5 μg/ml IBPG, 0.5 μg/ml IBP and 4 μg/ml I.S.

ma and plasma spiked with IBPG, IBP and I.S. No interfering peaks from endogenous compounds were observed when blank plasma was analyzed at either 225 or 214 nm. The retention times of IBPG, IBP and I.S. were 9.1, 24.8 and 16.1 min, respectively. Analysis of aspirin, acetaminophen and salicylic acid standard solutions and plasma samples spiked with the drugs demonstrated that these drugs did not interfere with the analysis as there were no interfering signals in the chromatogram after injection of standard solutions of these drugs at 100 μg/ml or extracts of plasma spiked with these over-the-counter drugs.

Extraction efficiency, linearity and variability

Table I shows the recovery of IBPG and IBP, respectively, by solid-phase extraction. Recoveries were greater than 55% from plasma over the concentration range 0.2–10 μg/ml for IBPG and 0.5–50 μg/ml for IBP. At the concentration employed, 4 μg/ml, the I.S. of ibufenac was extracted at greater than 70%. The calibration curves were linear over the range 0.1–10 μg/ml for IBPG and 0.5–100 μg/ml for IBP with correlation coefficients (r) >0.995 for both glucuronide and parent drug. Moreover, normalized response (peak-area ratio/concentration) was consistent across the range.

The intra-day variability of the method was determined by analysis of five plasma samples containing four different concentrations for IBPG and three concentrations for IBP on two different days. The results are presented in Table II. The coefficient of variation of the method ranged from 2.7 to 9.5 and 4.6 to 13% for IBP and IBPG, respectively. To determine the inter-day variability of the assay, the quality control samples at high, medium and low concentrations of IBPG (9.75, 1.95 and 0.49 μg/ml, respectively) and IBP (30, 5.55 and 0.56 μg/ml, respectively) were assayed in duplicate on several days (Table III).

The inter-day variability was also calculated from the slopes, standard deviation and variability of four standard curves for IBPG and IBP which were independently prepared. The slope values ±S.D. of IBP and IBPG were 0.229 ± 0.018 (C.V. = 7.86%) and 0.312 ± 0.014 (C.V. = 4.49%), respectively, over the range of 0.2–10 μg/ml for IBPG and 0.5–50 μg/ml for IBP.

The minimal concentration tested by the assay was 0.5 μg/ml for IBP and 0.2 μg/ml for IBPG with a within-day coefficient of variation of 5 and 7%, respectively. The use of a fixed-wavelength

TABLE I

EXTRACTION EFFICIENCIES OF IBUPROFEN GLUCURONIDE AND IBUPROFEN FROM PLASMA

Compound	Concentration (μg/ml)	Extraction (mean ± S.D., n = 5) (%)
IBPG	10	82.4 ± 1.5
	2	100.0 ± 2.1
	0.5	98.7 ± 4.5
	0.2	95.9 ± 6.8
IBP	25	55.9 ± 5.0
	5.0	71.3 ± 5.3
	0.5	74.8 ± 4.4

TABLE II

INTRA-DAY VARIABILITIES IN THE ANALYSIS OF PLASMA CONCENTRATIONS OF IBUPROFEN GLUCURONIDE AND IBUPROFEN ON TWO DIFFERENT DAYS

Concentration (μg/ml)	C.V. (n = 5) (%)	
	IBPG	IBP
Day 1		
25	–	3.6
10	8.8	–
5	–	2.7
2	6.7	–
0.5	6.7	5.0
0.2	9.0	–
Day 2		
25		9.5
10	4.6	–
5	–	9.4
2	13	–
0.5	5.0	5.9
0.2	7.7	–

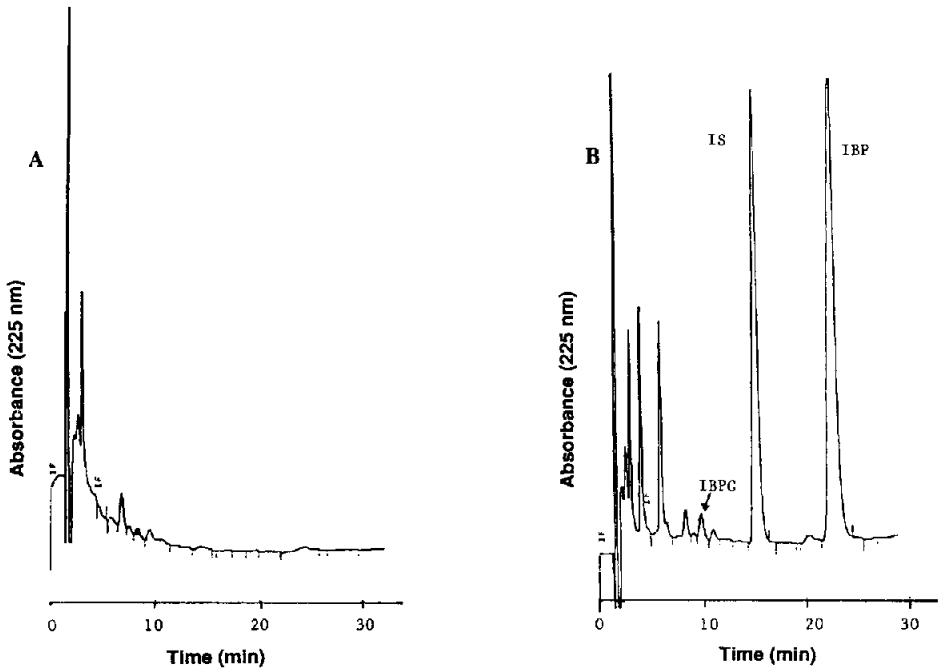

Fig. 3. Analysis of plasma samples from a volunteer (A) prior to the dose and (B) 7 h after oral administration of 800 mg of IBP. The plasma concentrations shown for IBPG and IBP after 7 h are 0.15 and 5.71 μg/ml, respectively.

detector at 214 nm was helpful to detect IBPG plasma concentration of 0.1 μg/ml with a within-day coefficient of variation of 12%.

TABLE III

INTER-DAY VARIABILITIES OF THE ASSAY METHOD FOR DETERMINING IBUPROFEN GLUCURONIDE AND IBUPROFEN IN PLASMA

Actual concentration (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Error[a] (%)	C.V. (%)
IBPG (n = 6)			
High	9.75	8.85 ± 0.8	9.2	9.2
Medium	1.95	1.83 ± 0.2	−6.2	11.4
Low	0.49	0.50 ± 0.04	2.0	8.0
IBP (n = 4)			
High	30.01	30.8 ± 3.0	2.6	9.9
Medium	5.55	6.0 ± 0.4	8.1	7.3
Low	0.56	0.51 + 0.07	−7.6	13.2

[a] Error = 100 × (concentration found − actual concentration)/actual concentration.

Application

Representative chromatograms of plasma samples analyzed from a volunteer collected prior to and 7 h after IBP administration are shown in Fig. 3. Fig. 4 shows the plasma concentration–

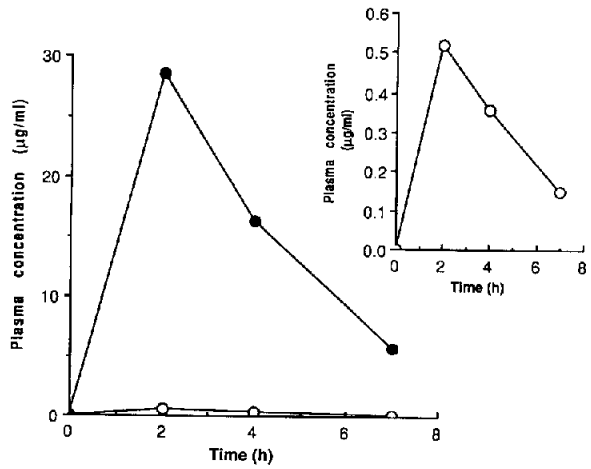

Fig. 4. Plasma concentration–time profile of IBPG (○) and IBP (●) from a volunteer after oral administration of 800 mg of IBP. The inset shows the plasma levels of IBPG which are presented as IBP equivalents.

time profiles of IBPG and IBP of this volunteer. The results indicate that the plasma levels of IBPG are around 2% of the IBP at every sampling time. The concentrations found during the study for both compounds were in the concentration range validated.

DISCUSSION

The data indicate that this analytical method for the determination of IBPG and IBP is adequate for disposition studies of IBP conjugation in humans. The validation parameters showed a quantitative recovery, minimal chromatographic background, good intra- and inter-day reproducibility, linearity over 0.5–100 μg/ml for IBP (concentrations observed after normal therapeutic doses), 0.1–10 μg/ml for IBPG, and limit of detection of 0.1 μg/ml for IBPG. Satisfactory column performance was maintained over a two-year period with frequent use. Though retention and efficiency of the column did decline slightly over several years, selectivity of the three compounds was unaltered.

This method utilized lower wavelengths of 225 and 214 nm for the detection of IBP and IBPG than previously employed (278 nm) by Liu *et al.* [21] for the analysis of urine. The use of lower wavelengths which improved detection limits also necessitated more selective sample extraction, as it was found that liquid–liquid extraction, as previously employed by Liu *et al.* [21], was not satisfactory when determining IBPG in plasma at concentrations below 1 μg/ml. The use of a washing step prior to elution of IBPG and IBP from the solid-phase extraction cartridge was essential in providing a chromatogram with less interference for IBPG at the low levels measured in plasma.

In order to elute both IBPG and the much more lipophilic IBP in a reasonable time without gradient elution, reduced selectivity of drug relative to metabolite was achieved using a mobile phase with TFA at pH 2. This low pH provided a selectivity of 1.6 for IBP/IBPG, very similar to the value of 1.32 obtained with a more complex mobile phase based upon ion-pairing with cetheexonium at pH 6 [21]. Moreover, acyl glucuronides as IBPG are more stable at low pH where acyl migration and transesterification are minimized [8,9,21].

Because acyl glucuronides are unstable and undergo intramolecular acyl migration under physiological conditions, precautions have to be taken to maintain an acidic pH in plasma samples in order to minimize the IBPG degradation during sample handling and storage. Such precautions have been mentioned by other authors as being essential for the accurate measurement of labile acyl glucuronides [8,9].

The applicability of the assay was demonstrated by a preliminary study of the plasma concentration–time profile of IBPG and IBP in a normal subject. The results indicate that the plasma levels of the acyl glucuronide metabolite are approximately 2% of those observed for IBP. Due to the low IBPG plasma concentrations relative to IBP, this direct analysis has advantages over indirect assay based upon measurement of the difference in IBP concentration after enzymatic or chemical hydrolysis. Although there is a report where IBP and its *R,S*-glucuronide are analyzed simultaneously in human liver microsomes by HPLC, it requires a gradient for the mobile phase and drug extraction from the sample was not necessary [23]. Because of the low IBPG plasma levels found in this study, a sensitive assay after an efficient and selective extraction of IBP and IBPG was required. Liquid–liquid extraction was attempted, but the organic solvents investigated extracted endogenous compounds which had retention times similar to the retention times of IBP and IBPG. Because the extraction efficiency and selectivity of the solid-phase extraction were particularly suitable and because solid-phase extraction had advantages over liquid–liquid extraction, the method reported here allowed simultaneous determination of both parent drug and polar metabolite.

In conclusion, the HPLC method presented is direct, simple, selective, reproducible, sensitive and linear for the simultaneous determination of IBPG and IBP in human plasma and is adequate for clinical studies. The assay is also applicable to

in vitro experiments, such as stability studies of IBPG and its covalent binding to proteins.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant GM 41828. P.C.S. is a recipient of a Faculty Development Award from the Pharmaceutical Manufacturers Association Foundation. The mass spectrometry of IBPG by Dr. Susan Weintraub is greatly appreciated.

REFERENCES

1 G. J. Dutton, *Glucuronidation of Drugs and other Compounds*, CRC Press, Boca Raton, FL, 1980, p. 268.
2 A. Gautam, H. Seligson, E. R. Gordon, D. Seligson and J. L. Boyer, *J. Clin. Invest.*, 73 (1984) 873.
3 A. F. McDonagh, L. A. Palma, J. J. Lauff and T. W. Wu, *J. Clin. Invest.*, 74 (1984) 763.
4 P. C. Smith, A. F. McDonagh and L. Z. Benet, *J. Clin. Invest.*, 77 (1986) 934.
5 M. L. Hyneck, P. C. Smith, A. Munafo, A. F. McDonagh and L. Z. Benet, *Clin. Pharmacol. Ther.*, 44 (1988) 107.
6 J. A. Watt and R. G. Dickinson, *Biochem. Pharmacol.*, 39 (1990) 1067.
7 G. E. McKinnon and R. G. Dickinson, *Res. Commun. Chem. Pathol. Pharmacol.*, 66 (1989) 339.
8 P. N. J. Langendijk, P. C. Smith, J. Hasegawa and L. Z. Benet, *J. Chromatogr.*, 307 (1984) 371.
9 P. C. Smith, J. Hasegawa, P. N. J. Langendijk and L. Z. Benet, *Drug Metab. Dispos.*, 13 (1985) 110.
10 G. Geisslinger, K. Dietzel, D. Loew, O. Schutser, G. Lachmann and K. Brune, *J. Chromatogr.*, 491 (1989) 139.
11 J. H. G. Jonkman, R. Schoenmaker, A. H. Holtkamp and J. Hempenius, *J. Pharm. Biomed. Anal.*, 3 (1985) 433.
12 A. Shah and D. Jung, *J. Chromatogr.*, 344 (1985) 408.
13 A. Avgerinos and A. J. Hutt, *J. Chromatogr.*, 380 (1986) 468.
14 M. Lalande, D. L. Wilson and J. McGilveray, *J. Chromatogr.*, 377 (1986) 410.
15 P. J. Streete, *J. Chromatogr.*, 495 (1989) 179.
16 J. H. Satterwhite and F. D. Boudinot, *J. Chromatogr.*, 497 (1989) 330.
17 M. C. Nahata, *J. Liq. Chromatogr.*, 14 (1991) 187.
18 A. M. Rustum, *J. Chromatogr. Sci.*, 29 (1991) 16.
19 A. Shah and D. Jung, *J. Chromatogr.*, 378 (1986) 232.
20 B. Chai, P. E. Minkler and C. L. Hoppel, *J. Chromatogr.*, 430 (1988) 93.
21 H. F. Liu, P. Leroy, A. Nicolas, J. Magdalou and G. Siest, *J. Chromatogr.*, 493 (1989) 137.
22 K. A. Sinclair and J. Caldwell, *Biochem. Pharmacol.*, 31 (1982) 953.
23 M. El Mouelhi, H. W. Ruelius, C. Fenselau and D. M. Dulik, *Drug Metab. Dispos.*, 15 (1987) 767.